

Type I IFN Modulates the Immune Response Induced by DNA Vaccination to Pseudorabies Virus Glycoprotein C

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DNA vaccines have the capacity to induce strong Th1-biased immune responses that are of major importance to providing protection against intracellular pathogens. In the present study we have focused on the role played by type I IFN in immune responses induced after DNA vaccination. Mice lacking the IFNAR1 chain of the type I IFN receptor (IFNAR K/O mice) were immunized with a plasmid encoding glycoprotein C of pseudorabies virus (PRV-gC). After DNA vaccination, wild-type (WT) mice showed features characteristic of Th1 immune responses, such as high IgG2a:IgG1 anti-PRV Ab ratio and antigen-specific IFN- γ production by spleen cells. In contrast, IFNAR K/O mice showed a significantly lower IgG2a:IgG1 Ab ratio and IFN- γ production. In addition, the percentage of CD8⁺ and B lymph-node cells expressing CD69 after PRV-gC DNA vaccination was lower in IFNAR K/O than in WT mice. These results support a major role played by type I IFN in shaping Th1 immune responses after DNA vaccination. Codelivery of plasmids encoding IL-12 and IL-18 along with the plasmid encoding PRV-gC restored Th1 responses in IFNAR K/O mice. © 2001 Academic Press

Key Words: DNA vaccination; type I IFN; pseudorabies virus; Th1 immune response.

INTRODUCTION

Direct injection of plasmid DNA encoding protective antigens has been shown to induce both humoral and cellular immune responses as well as protection against infection, emphasizing the interest of this novel approach to vaccination (for reviews see Gurunathan *et al.*, 2000; Babiuk *et al.*, 1999; Tighe *et al.*, 1998). One important advantage of DNA vaccines is their ability to allow antigen presentation by both MHC class I and II molecules (Whitton *et al.*, 1999). Since successful induction of immune responses generally requires the presence of adjuvants, another advantage of DNA vaccines is that they contain specific nucleotide sequences that act as adjuvants. It is indeed well established that bacterial DNA and synthetic oligonucleotides containing unmethylated CpG motifs cause strong activation of B cells, T cells, NK cells, macrophages, and dendritic cells (Krieg *et al.*, 1995; Sun *et al.*, 1998; Asakura *et al.*, 2000; Hartman *et al.*, 1999; Jakob *et al.*, 1998; Sparwasser *et al.*, 1998).

Th1 specific effector functions were shown to be important for protective immune responses against intracellular pathogens, including viruses (Ramshaw *et al.*, 1997). Therefore understanding the mechanisms involved in Th1 polarization of the immune response after DNA vaccination is an important issue for development of antiviral vaccines. The potent immunostimulatory activity of bacterial DNA controlled by unmethylated CpG

dinucleotide motifs may have important implications for priming and maintenance of Th1-type immune responses (Chu *et al.*, 1997). These unmethylated CpG sequences augment the expression of costimulatory molecules on antigen-presenting cells (APC) and trigger them to synthesize cytokines such as IL-12, IL-18, and type I IFN (Sato *et al.*, 1996; Roman *et al.*, 1997; Jakob *et al.*, 1999). The ability of APC to present antigens to Th cells is therefore enhanced and the cytokines they release can contribute to bias Th differentiation toward Th1. The major role played by IL-12 and IL-18 in the shaping of Th1 responses is well established (Okamura *et al.*, 1998). Type I IFN plays a major role in T- and B-cell activation triggered by unmethylated CpG motifs (Sun *et al.*, 1998). However the way type I IFN acts on lymphocytes is still unclear (Sun and Sprent, 2000). In addition, the possible adjuvant role of type I IFN on antigen-specific immune responses to DNA vaccination has not been investigated.

To evaluate *in vivo* the effects of type I IFN on antiviral immune responses induced by DNA vaccination, we took advantage of mice deficient in a functional type I IFN receptor (IFNAR1) as an animal model (Muller *et al.*, 1994). The viral model we used is pseudorabies virus (PRV), a member of the alphaherpesvirus subfamily, responsible for economically important diseases in domestic animals (Mettenleiter, 1996). Among several viral envelope antigens, glycoprotein C (gC) was shown to be important in the initiation of the immune response and to act as a major target for cytotoxic T-lymphocytes (Zuckermann *et al.*, 1990).

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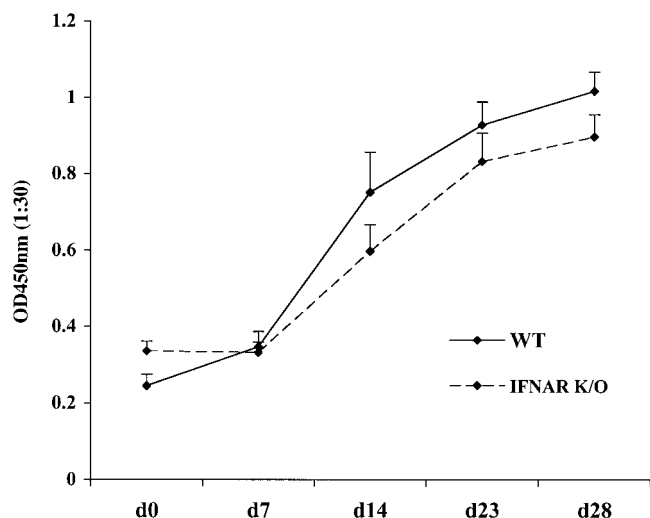


FIG. 1. Anti-PRV IgG Ab production in IFNAR K/O mice compared with WT after PRV-gC DNA vaccination. PRV-specific IgG levels were measured by ELISA. Each group of mice ($n = 5$) was immunized id with 100 μ g of VR1055-gC at day 0 and 14. Mice were bled each week and the sera were diluted 1:30 for ELISA. Data represent Ab levels (OD at 450 nm) WT mice (\diamond — \diamond); IFNAR KO mice (\diamond --- \diamond). Values and bars represent mean and SEM for five mice. Results from one representative experiment of a group of 2 are shown.

In the present study, several criteria of the immune response induced by a plasmid coding for PRV-gC were evaluated in type I IFN receptor knockout mice. The parameters tested included the specific isotype antibody response (IgG1 vs IgG2a) and IFN- γ synthesis after *in vitro* antigen-specific restimulation as markers of Th1 responses (Mosmann and Coffman, 1989). To investigate the activation of T and B cells following DNA vaccination, the expression of CD69 (an early activation marker) was monitored by flow cytometry analysis. Our results show that type I IFN was involved in the shaping of a Th1-type immune response to DNA vaccine encoding the PRV-gC.

RESULTS

PRV-gC DNA vaccination triggers antigen-specific antibody production in IFNAR K/O and wild-type (WT) mice

Mice received 100 μ g of DNA encoding PRV-gC (VR1055-gC) administered id in both ears on days 0 and 14. The IgG antibody response was monitored by PRV-specific ELISA in serum samples from IFNAR K/O and WT mice. In both mouse strains anti-PRV-IgG production increased after day 7 and reached its maximum 1–2 weeks after booster injection (Fig. 1). The amounts of anti-PRV-IgG were similar at all time points in IFNAR K/O and WT mice ($P > 0.05$) (Fig. 1). This implies that both mouse strains exhibited the ability to respond to DNA-encoded PRV-gC.

Reduced IgG2a:IgG1 anti-PRV antibody ratio in IFNAR K/O mice

The amount of IgG2a and IgG1 Ab produced upon vaccination with VR1055-gC was assayed by PRV-specific ELISA in serum samples. WT mice exhibited significantly higher levels of anti-PRV-IgG2a than of anti-PRV-IgG1 Ab after the first plasmid injection ($P < 0.01$ at day 14) and after the booster injection ($P < 0.001$ at days 23 and 27) (Fig. 2A). By contrast, IFNAR K/O mice raised similar levels of anti-PRV-IgG2a and anti-PRV-IgG1 Ab after the first and second plasmid injection (Fig. 2B). Accordingly, IgG2a:IgG1 ratios were significantly lower in IFNAR K/O than in WT mice (1.92 vs 6.71 at day 23, $P < 0.01$) (Fig. 2). These data were obtained with OD measured in 1:30 diluted sera. Similar differences were also observed with OD measured in 1:15 or 1:45 diluted sera,

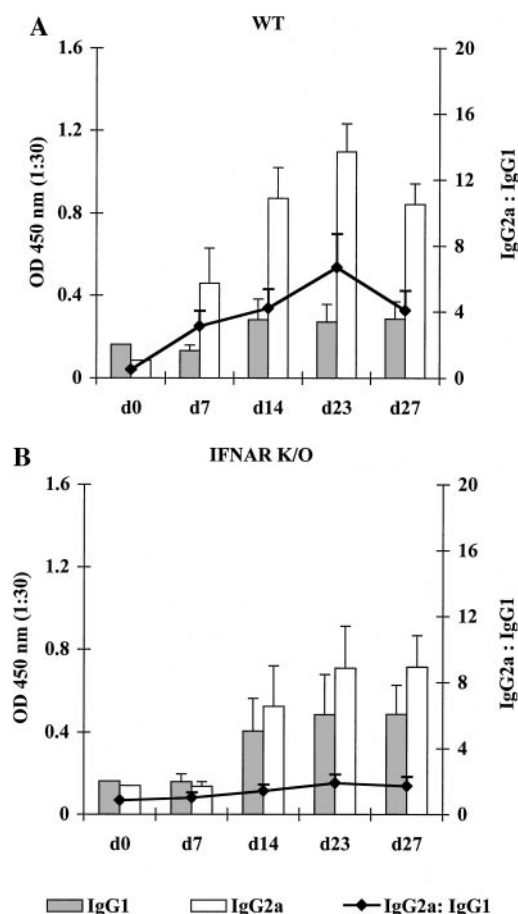


FIG. 2. Reduced IgG2a Ab level and IgG2a:IgG1 Ab ratio in IFNAR K/O mice compared to WT mice after PRV-gC DNA vaccination. PRV-specific IgG1 (\square) and IgG2a (\square) levels were measured by ELISA. Each group of mice ($n = 5$) was immunized id with 100 μ g of VR1055-gC at day 0 and 14. Mice were bled each week and the sera were diluted 1:30 for ELISA. Data represent Ab levels (OD at 450 nm) and IgG2a:IgG1 ratio (\diamond — \diamond). (A) WT mice. (B) IFNAR K/O mice. Values and bars represent mean and SEM for five mice. Results from one representative experiment of a group of 5 are shown.

or with Ab titers calculated by limiting dilutions (not shown). The high ratio of IgG2a to IgG1 Ab seen in WT mice indicates that PRV-gC DNA vaccination elicited a bias toward Th1 immune responses. The reduced IgG2a:IgG1 Ab ratio observed in K/O mice suggests that type I IFN contributed to the shaping of Th1 responses.

PRV-restimulated splenocytes of vaccinated IFNAR K/O mice synthesized less IFN- γ

To test the hypothesis of a role for type I IFN in shaping Th1 responses, we have compared IFN- γ production by splenocytes of IFNAR K/O and WT mice inoculated twice with VR1055-gC. IFN- γ production was quantified by ELISA in the supernatant of splenocytes cultured 48 h with PRV antigen. The amount of IFN- γ secreted by splenocytes of both mouse strains was significantly increased ($P < 0.0001$ in WT and $P < 0.05$ in IFNAR K/O mice) after VR1055-gC vaccination as compared to nonvaccinated mice (Figs. 3A and 3B). However following VR1055-gC inoculation, WT splenocytes produced 3.6 times more IFN- γ after *in vitro* restimulation with PRV antigens than IFNAR K/O splenocytes ($P < 0.0001$) (Figs. 3A and 3B). Thus, in the absence of a functional type I IFN receptor, splenocytes of VR1055-gC vaccinated mice have a reduced ability to secrete IFN- γ .

The percentage of CD8⁺ and B lymph-node cells expressing CD69 following VR1055-gC vaccination is reduced in IFNAR K/O mice

As type I IFN are known to affect the expression of activation markers on T and B cells (Sun and Sprent, 2000; Sun *et al.*, 1998), we have assessed the level of CD69 (early activation antigen) expression by T and B lymph-node cells. Draining lymph-node cells of both mouse strains were stained with anti-CD69 mAb in combination with anti-CD8, anti-CD4, or anti-slg mAbs at 18 and 48 h after VR1055-gC injection and analyzed by flow cytometry.

VR1055-gC injection significantly increased the percentage of CD8⁺ and B cells expressing CD69 at 18 h ($P < 0.02$ and $P < 0.0002$, respectively) in draining lymph nodes of WT mice but not of IFNAR K/O mice (Figs. 4A and 4B). By contrast the percentage of CD4⁺ cells expressing CD69 in both mouse strains did not significantly increase upon VR1055-gC injection (Figs. 4A and 4B). The differential pattern of CD69 expression by T and B cells is shown for one individual (Fig. 5).

Thus, our results show that early activation of CD8⁺ T and B cells (shown by CD69 expression) in response to DNA-encoded PRV-gC required a functional type I IFN signaling pathway.

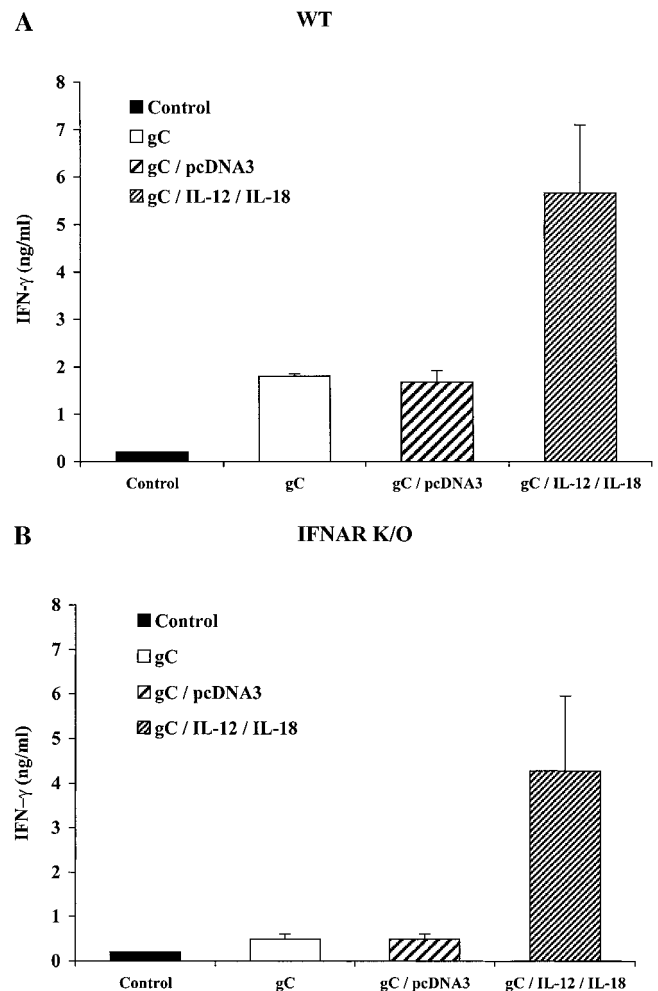


FIG. 3. Reduced IFN- γ production by PRV-restimulated IFNAR K/O splenocytes. Mice were immunized id with 100 μ g of VR1055-gC at day 0 and 14. Some mice were coinjected with IL-12 and IL-18 plasmids (gC/IL-12/IL-18) or with empty plasmid (gC/pcDNA3) as a negative control for IL-12/IL-18. Control mice were not injected. Spleens were collected 2 weeks after booster injection and cell cultures were stimulated with either PRV antigen or culture medium as negative control, or PMA and ionomycin as a positive control. The level of IFN- γ was measured 48 h later by ELISA. IFN- γ levels were <0.2 ng/ml in negative controls and >20 ng/ml in positive controls. One of three representative experiments is shown. (A) WT mice; (B) IFNAR KO mice.

Codelivery of IL-12 and IL-18 encoding plasmids restored Th1 responses but not CD69 expression in VR1055-gC-vaccinated IFNAR K/O mice

Plasmids encoding IL-12 and IL-18 (pWRG3169-mIL-12 and pCR3.1::mIL-18) were coinjected with VR1055-gC to IFNAR K/O mice to test whether Th1 responses (IgG2a:IgG1 ratio and IFN- γ production) could be restored by IFN- γ -promoting cytokines.

IL-12/IL-18 DNA coinjection significantly enhanced IFN- γ production by *in vitro* PRV-restimulated splenocytes from both IFNAR K/O and WT mice as compared to mice injected with VR1055-gC only (Fig. 3A, $P < 0.001$, and Fig. 3B, $P < 0.02$). Furthermore after IL-12 and IL-18 DNA coinjection, spleno-

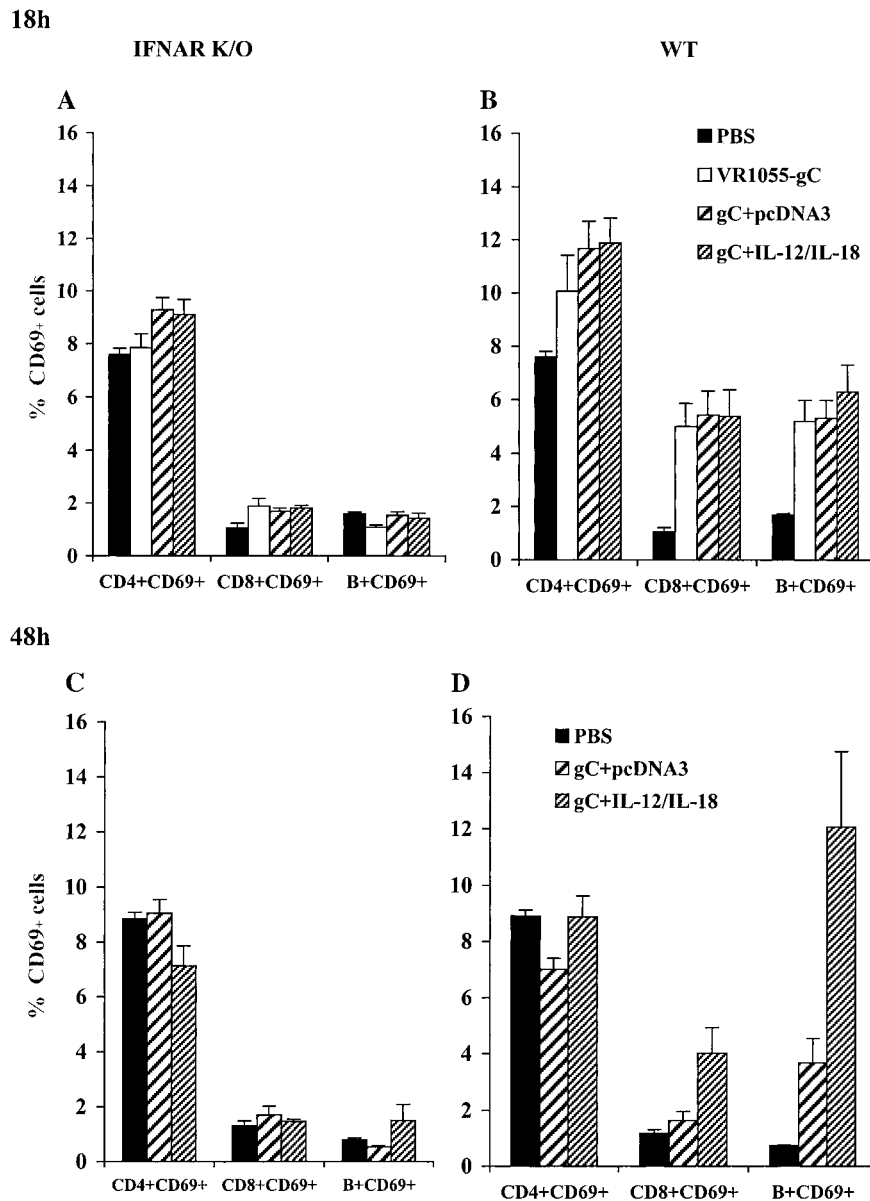


FIG. 4. The percentage of CD8⁺ and B lymph-node cells expressing CD69 is lower in PRV-gC-vaccinated IFNAR K/O as compared to WT mice even after codelivery of IL-12/IL-18 plasmids. Each group of mice ($n = 6$) was injected id with 100 μ g of VR1055-gC, except control mice ($n = 6$), which were injected with PBS. One group of mice was coinjected with IL-12 and IL-18 plasmids (gC/IL-12/IL-18); another group was injected with empty plasmid (gC/pcDNA3) as a negative control for IL-12/IL-18. 18 h later (Figs. 4A and 4B) and 48 h later (Figs. 4C and 4D) draining lymph-node cells were double-stained for CD4, CD8, slg, and CD69. Values and bars represent mean and SEM for six mice. (B, D) WT; (A, C) IFNAR K/O mice.

cytes of IFNAR K/O and WT mice displayed equivalent ability to produce IFN- γ (Fig. 3B compared to Fig. 3A).

Coinjection of the plasmids coding for IL-12/IL-18 to VR1055-gC-vaccinated IFNAR K/O mice increased IgG2a and decreased IgG1 Ab levels (Fig. 6A compared to Fig. 2B). Thus, whereas IgG2a:IgG1 Ab ratios were always < 2 in VR1055-gC-vaccinated IFNAR K/O mice (Fig. 2B), they increased up to 8 in IL-12/IL-18 injected mice (Fig. 6A). Codelivery of IL-12/IL-18 DNA to WT mice also resulted in an increase of IgG2a Ab and the IgG2a:IgG1 Ab ratio (Fig. 6B compared to Fig. 2A).

The percentage of CD8⁺ and B cells expressing CD69 in the draining lymph node of IFNAR K/O mice was not increased upon coinjection of IL-12/IL-18 DNA with VR1055-gC at 18 and 48 h (Figs. 4A and 4C). However when administered to WT mice, IL-12/IL-18 DNA significantly increased the percentage of CD8⁺ and B cells expressing CD69 at 48 h (Fig. 4D, $P < 0.05$ and $P < 0.01$, respectively) but not at 18 h (Fig. 4B). At both time points the percentage of CD69⁺ CD4⁺ was not significantly altered by IL-12/IL-18 DNA codelivery (Figs. 4B and 4D).

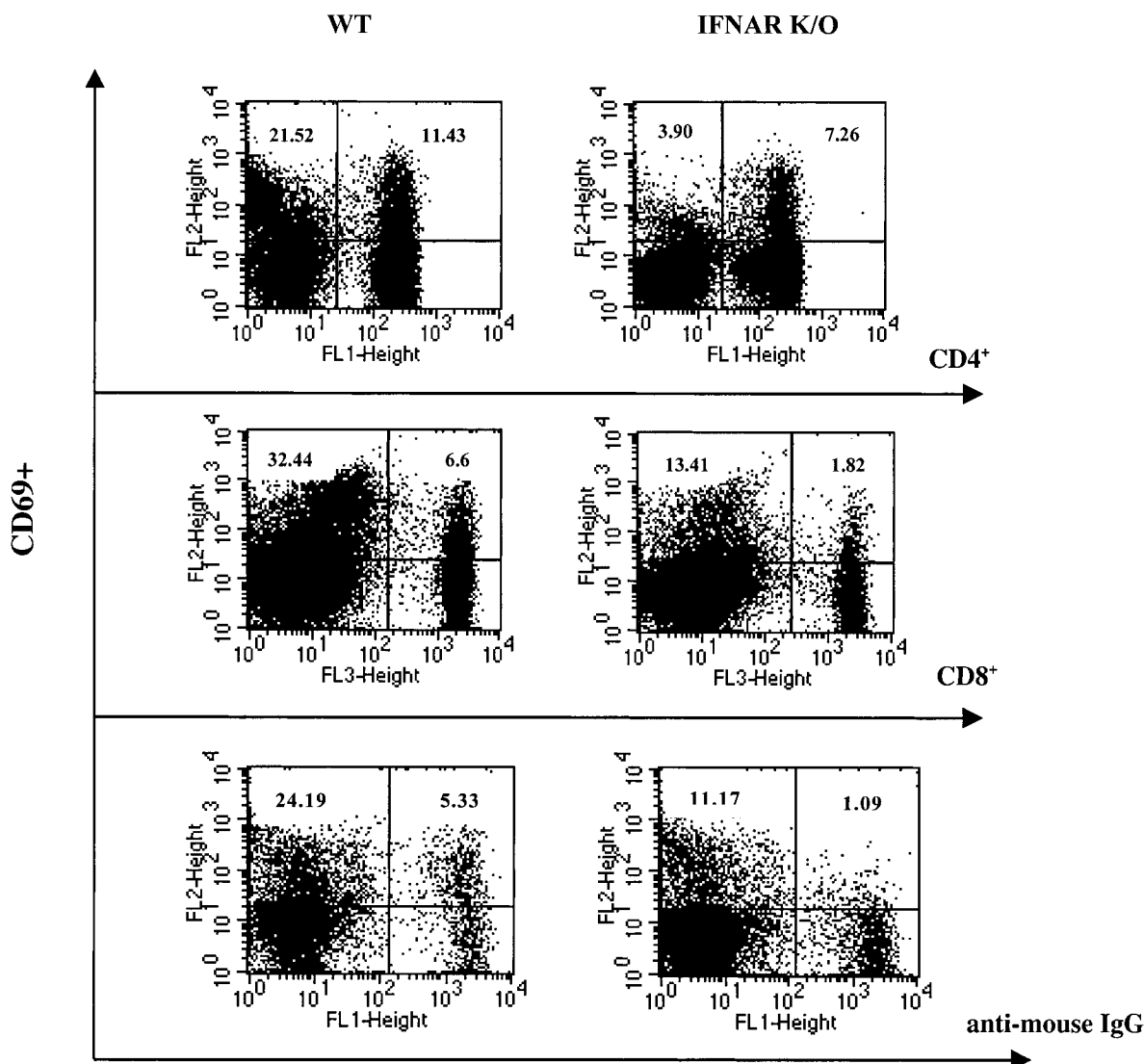


FIG. 5. Differential pattern of expression of CD69 by T and B cells between IFNAR K/O and WT mice. Each group of mice (seven WT and eight IFNAR K/O) was injected id with 100 μ g of VR1055-gC; 18 h later, draining lymph-node cells were double-stained for CD4, CD8, slg, and CD69. Double staining for CD69 (FL2) and CD4 (FL1) or CD8 (FL3) or B cells (FL1) is shown. Quadrant limits were set according to matching isotype dot plots. The upper windows contain the CD69-positive cells. The percentage of single- and double-positive cells are shown for each dot plot. Dot plots from one representative individual are shown.

An empty plasmid (pcDNA3) was coinjected with VR1055-gC instead of the IL-12 and IL-18 coding plasmids to control the specificity of their effect. The empty plasmid codelivered with VR1055-gC had no effect on IFN- γ production (Fig. 3) but did increase the level of IgG1 and IgG2a Ab in both mouse strains (Figs. 6C and 6D vs Fig. 2) without affecting the IgG2a:IgG1 ratio. Therefore the adjuvant effect of the control plasmid on total Ab production (presumably due to CpG motifs) (Chu *et al.*, 1997) was significantly different from that exerted by IL-12/IL-18 coding plasmids. The empty plasmid codelivered with VR1055-gC instead of IL-12/IL18 DNA had no effect on the percentage of WT lymph-node cells expressing CD69 (Figs. 4B and 4D).

DISCUSSION

To gain insight into the role played by type I IFN in DNA vaccination to viral antigens, we examined antigen-specific immune responses triggered by DNA encoding PRV-gC in WT and IFNAR K/O mice. PRV-gC DNA vaccination of WT mice induced antigen-specific IFN- γ production by splenocytes and a high ratio of IgG2a:IgG1 anti-PRV Ab in serum. These features indicated a bias toward Th1 immune responses. In the absence of a functional type I IFN signaling pathway, PRV-gC DNA immunization resulted in a much lower IgG2a:IgG1 Ab ratio and decreased IFN- γ production by splenocytes. These differences between IFNAR K/O and WT mice immune responses could reflect a different availability of

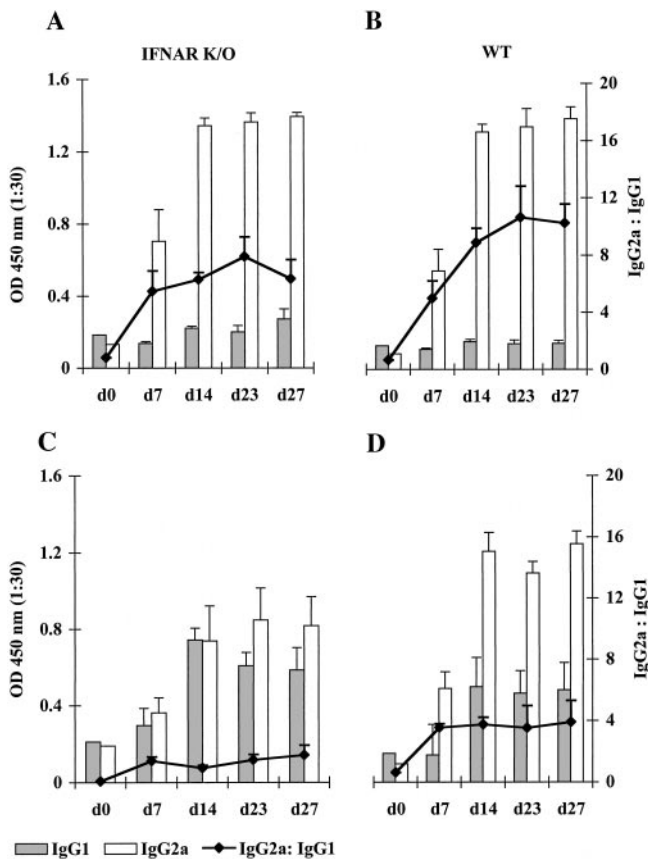


FIG. 6. Codelivery of IL-12/IL-18 plasmids enhanced the IgG2a Ab level and IgG2a:IgG1 Ab ratio in IFNAR K/O mice immunized with PRV-gC DNA. PRV-specific IgG1 (■) and IgG2a (□) levels were measured by ELISA. Each group of mice ($n = 5$) (B, D: WT; A, C: IFNAR K/O mice) was immunized id with 100 μ g of VR1055-gC and coinjected with IL-12 and IL-18 plasmids (A and B) or with empty plasmid (C and D) at day 0 and 14. Mice were bled each week and the sera were diluted 1:30 for ELISA. Data represent Ab levels (OD at 450 nm) and IgG2a:IgG1 ratio (◆—◆). Values and bars represent mean and SEM for five mice. Results from one representative experiment out of five is shown.

PRV-gC antigen. However the observation that the total IgG Ab response did not differ between the two strains of mice suggested that IFNAR K/O and WT mice have similar ability to take up the DNA-encoded viral antigen and then transcribe/translate it. Therefore the reduced ability of IFNAR K/O mice to generate Th1 responses to PRV-gC DNA points to a regulatory role for type I IFN in the process leading to Th1-biased immune responses.

To understand the pathways by which type I IFN favors a bias toward Th1 immune responses, we investigated early T- and B-cell activation in the lymph node draining the site of PRV-gC DNA injection. Indeed, DNA or CpG motifs are known to induce up-regulation of cell adhesion/activation molecules including CD69 and B7-2 in a process requiring type I IFN (Kranzer *et al.*, 2000; Sun *et al.*, 1998). CD69 is an activation marker expressed shortly after lymphocyte activation and acts as a costimulatory molecule (Borrego *et al.*, 1999). DNA vaccination of WT mice resulted in a rapid increase in the percentages of

CD8⁺ and B lymphocytes expressing CD69. By contrast, the percentage of CD8⁺ and B lymph-node cells expressing CD69 in IFNAR K/O mice remained at basal levels. The percentage of CD4⁺ cells expressing CD69 was only moderately increased and there was no significant difference between WT and IFNAR K/O mice. Therefore CD8⁺ and B cells were the first subsets of lymphocytes displaying an activated phenotype in regional lymphoid tissues and type I IFN played a critical role in this early process. The consequences of rapid activation of CD8⁺ and B cells for shaping Th1 responses may be of two sorts.

First, activated CD8⁺ T cells could be a major early source of IFN- γ that will subsequently contribute to the differentiation of Th0 into Th1 cells. In fact in mice, in contrast to humans, the type I IFN signaling pathway (STAT4) leading Th cells to produce IFN- γ is not functional. In contrast, murine CD8⁺ T cells were found to produce IFN- γ in the absence of STAT4 activation (Farrar and Murphy, 2000). Therefore, a STAT4-independent pathway involving CD8⁺ T-cell activation by type I IFN could provide the cytokine microenvironment allowing Th cells differentiation into Th1 cells.

Second, type I IFN may act directly on B cells. Previous work using anti-IgD immunization in mice has shown that type I IFN regulates Ig-isotype selection through a direct effect on B cells (Finkelman *et al.*, 1991). Moreover CpG DNA *in vivo* was shown to induce B cells to proliferate and secrete Ig (Krieg *et al.*, 1995). We propose therefore that type I IFN supported a predominant IgG2a Ab response after PRV-gC DNA vaccination by directly activating B cells (proliferation and isotype selection).

Since IL-12 and IL-18 are known to favor Th1 orientation of immune responses (Okamura *et al.*, 1998), we investigated in our experimental conditions whether the administration of plasmids encoding IL-12 and IL-18 would restore Th1 responses. IL-12/IL-18 DNA delivery together with PRV-gC DNA strongly increased the IgG2a:IgG1 Ab ratio and PRV-specific IFN- γ production by splenocytes in IFNAR K/O mice and to a lesser extent in WT mice. Thus IL-12/IL-18 DNA codelivery has restored the antigen-specific-Th1 responses in IFNAR K/O mice. These results are in accordance with other studies showing an enhancement of IFN- γ secretion after injection of plasmids encoding IL-12 (Sin *et al.*, 1999) or IL-18 (Kremer *et al.*, 1999). In addition, in some models IL-12 codelivery increased the IgG2a:IgG1 ratio (Chow *et al.*, 1998), while in other models IL-12 or IL-18 induced an overall decrease of Ab production (Kim *et al.*, 1997).

A first explanation for the effect of IL-12 and IL-18 observed in our experimental model is their ability to directly trigger Th0 cell differentiation into Th1. IL-12/IL-18 DNA codelivery did not increase the percentage of CD8⁺ and B cells expressing CD69 in IFNAR K/O mice, although the percentage increased in WT mice at 48 h. This suggested that in our experimental model IL-12 and

IL-18 could restore Th1 responses without restoring the pathway leading to early CD8⁺ and B cell activation. Therefore we propose that type I IFN sustains Th1 responses through its action on CD8⁺ and B cells, whereas IL-12 and IL-18 can act directly on CD4⁺ cells. However we did not observe any increase in the percentage of CD4⁺ cells expressing CD69 after IL-12/IL-18 DNA codelivery in both WT and IFNAR K/O mice.

The codelivery of IL-12 and IL-18 DNA *in vivo* may have compensated for deficient synthesis of these cytokines in IFNAR K/O mice, although we have not formally proven the latter. However this hypothesis is not sustained by other *in vivo* studies based on inoculation of live virus (LCMV) or inactivated virus particles (HSV1), showing that type I IFN inhibits or does not modify IL-12 production, respectively (Cousens *et al.*, 1997; Riffault *et al.*, 2000).

The IFNAR1 chain of the type I IFN receptor was shown to facilitate the recruitment of IFN- γ -activated transcription factors (Takaoka *et al.*, 2000). Therefore in the absence of the IFNAR1 chain in IFNAR K/O mice, higher concentrations of IFN- γ may be required to efficiently trigger the IFN- γ receptor-signaling pathway. IL-12 and IL-18 DNA codelivery with PRV-gC DNA could sufficiently increase the amount of IFN- γ locally available in IFNAR K/O mice to restore IFN- γ -mediated responses.

In conclusion our experiments show that type I IFN plays an important role in Th1 polarization of the immune response induced by DNA vaccination to a viral protein. Our results imply therefore that tailoring of DNA sequences involved in type I IFN induction will constitute a key parameter for the improvement of DNA vaccines by enhancing Th1 responses. This is of major importance for protection against intracellular pathogens, especially viruses.

MATERIALS AND METHODS

Mice

Eight- to ten-week-old female pathogen-free mice, either deficient in type I IFN receptor function as a result of inactivation of the IFNAR 1 gene (IFNAR K/O, or A129) or wild-type 129SvEv control mice (WT, or 129), were both originally purchased from B&K Universal Limited (North Humberside, U.K.). Mice were bred within the INRA animal care facilities (Jouy-en-Josas, France) and handled in accordance with institutional guidelines for animal care and use.

Plasmids

A plasmid (VR1055-gC) driving the expression of PRV-gC protein under the control of the HCMV promoter was used in this study. A plasmid construct (kindly provided by Dr. André Jestin, AFSSA, Ploufragan, France) containing a 4.1-kb *Pst*I fragment of PRV (NIA3 strain)

genomic DNA was used as source of gC coding sequence (GenBank Accession No. D49437). The 1511-bp *Dra*I-*Hin*II fragment bearing the complete gC reading frame was purified and inserted in the proper orientation at the *Eco*RV site of the VR1055 expression vector (kindly provided by Dr. Robert Zaugg, Vical, San Diego, CA). Expression of gC was verified by flow cytometry analysis on transfected mouse fibroblast cells.

Plasmids pWRG3169-mIL-12 (obtained from Dr. A. L. Rakhmievich, University of Wisconsin, Madison, WI) and pCR3.1::IL-18 (provided by Dr. C. Loch, Pasteur Institute, Lille, France) allowed HCMV promoter-controlled expression of murine IL-12 (p35 and p40 subunits) and IL-18, respectively (Rakhmievich *et al.*, 1996; Kremer *et al.*, 1999). All plasmids were amplified in *Escherichia coli* DH5 α and purified by standard cleared lysate method, phenol chloroform extraction, and two rounds of banding by centrifugation on cesium chloride-ethidium bromide gradient (Sambrook *et al.*, 1989).

DNA vaccination protocol

Mice were inoculated *id* on day 0 and 14 in the dorsal side of each ear with 50 μ g VR1055-gC in a volume of 25 μ l using a 22-G needle (100 μ g VR1055-gC delivered in total) as described (Casares *et al.*, 1997; Riffault *et al.*, 2000). In the experiments where mice were coinjected with several plasmids, 50 μ g of each plasmid, coding for IL-12 and IL-18, were mixed prior to injection with 100 μ g of VR1055-gC. In these experiments control mice were inoculated with 100 μ g of the empty vector pcDNA3 (Invitrogen Groningen, The Netherlands) mixed prior to injection with 100 μ g of VR1055-gC.

Antigen-specific *in vitro*-restimulation of splenocytes

Injected mice were anesthetized with methoxyflurane and after sacrifice by cervical dislocation their spleens were harvested. Spleen cells, after RBC lysis, were cultured at 2×10^6 cells/ml in 96-well flat-bottomed microtiter plates (Falcon 3072) and stimulated with an m.o.i. of 10 TCID₅₀ of the vaccine PRV strain Bartha, kindly provided by Dr. A. Jestin (AFSSA, Ploufragan, France). For positive controls, spleen cells were treated with PMA (10 ng/ml, Sigma) and ionomycin (1 μ g/ml, Calbiochem). Supernatants were harvested from cultures after incubation at 37°C in 5% CO₂ for 48 h and frozen at -20°C until titration.

IFN- γ ELISA

Culture supernatants were tested in duplicate for IFN- γ using specific ELISA. Microtiter plates (Immulon, 2HB) were coated with 100 μ l anti-IFN- γ capture Ab (R4-6A2, PharMingen) at a concentration of 4 μ g/ml overnight at 4°C. The plates were washed five times with PBS/Tween and blocked with 100 μ l per well of 2% bovine serum albumin in PBS for 2 h at 37°C. Then the

plates were washed five times and incubated with samples and standard (recombinant murine IFN- γ , from Genzyme) overnight at 4°C. One hundred microliters of biotinylated anti-IFN- γ (XMG1.2, PharMingen) at a concentration of 1 μ g/ml was added to each well and incubated at 4°C for 3 h. The plates were then washed five times, 100 μ l of streptavidine-horseradish peroxidase (ImmunoPure Pierce, 1 μ g/ml) was added, and the mixture was incubated at 4°C for 1 h. Following washing, the substrate was added and absorbance was measured using an ELISA plate reader. The concentration of IFN- γ in the samples was determined by comparison with recombinant murine IFN- γ .

Flow cytometry analysis of CD69 expression by CD4, CD8, and B cells

After injection of the plasmids id, mouse auricular lymph nodes were harvested and cells were prepared by lymph-node disruption; 1×10^6 cells were distributed in 96-well flat-bottomed microtiter plates (Falcon 3072). Cells were double stained for 30 min on ice with PE-conjugated anti-CD69 (clone H1.2F3) (PharMingen) in combination with FITC-conjugated anti CD4 (clone CT-CD4), TC-conjugated anti CD8 (clone CT-CD8a) (Caltag), or biotin-conjugated goat anti-mouse IgG (Sigma) followed by FITC-conjugated streptavidin (PharMingen). Matching isotype controls were used in each case. Data were collected on 30,000 cells using a FACScan (Becton-Dickinson) flow cytometer and analysis was performed on CellQuest software (Becton-Dickinson). Cells were electronically gated according to light scatter properties to exclude cell debris.

Isotype-specific antibody assay

Serum samples were collected by retro-orbital bleeding at different time points and analyzed for the presence of PRV-specific Abs by ELISA. Briefly, microtiter plates (Falcon 3915, Becton-Dickinson) were coated with PRV antigen (Kaplan strain: concentrated and inactivated antigen preparation, kindly provided by Dr. J.-C. Audonnet, Merial, Lyon, France) at 0.08 mg/ml in carbonate-bicarbonate buffer (0.1 M, pH 9.5) overnight at 4°C. After incubation with 150 μ l of 0.5% bovine serum albumin in PBS/Tween for 1 h at 37°C, 100 μ l of prediluted test sera (1:30) was added in duplicate and incubated for 2 h at 37°C. After washing, Abs were detected with horseradish peroxidase-conjugated rat anti-mouse IgG (1:4000, Biosys), IgG1, or IgG2a (1:2000, PharMingen). Enzymatic reaction was developed by adding 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories Inc.) as a substrate. The absorbance was measured at 450 nm after addition of 1 M phosphoric acid.

Data analysis

All values were expressed as mean and standard error of the mean (SEM). Statistical analysis of the experimental data and controls was conducted by Student's *t* test. Significance was defined at $P < 0.05$ in statistical analysis.

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